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Dietary copper effects survival of channel catfish challenged with *Flavobacterium columnare*

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Abstract

Columnaris disease is an important bacterial disease of commercially grown channel catfish, *Ictalurus punctatus*. Copper sulphate ($CuSO_4$) has been shown to be therapeutic and prophylactic as a water treatment for columnaris disease. Copper is an essential dietary component in animal feeds and $CuSO_4$ is typically included in base diets; a study was conducted to evaluate whether fish feed supplemented with additional $CuSO_4$ at 0, 40 and 80 mg kg^{-1} of diet and fed at a daily rate of 3% body weight would affect survival to columnaris disease. Results indicate fish fed the copper-supplemented diet for 2 weeks significantly increased survival following *F. columnare* challenge. This increase appeared to be dose-dependent. The mean per cent survival (±SEM) for fish fed the base diet (unsupplemented) for 2 weeks and then challenged was 2.0% ± 1.1. Fish fed the base plus 40 mg $CuSO_4$ kg^{-1} had a mean survival of 22.0% ± 11.0. Fish fed the base plus 80 mg $CuSO_4$ kg^{-1} had a mean survival of 29.3% ± 13.4. The mean per cent survival for fish fed the base diet for 4 weeks and then challenged was 28.3% ± 9.0. Fish fed the base plus 40 mg $CuSO_4$ kg^{-1} for 4 weeks had a mean survival of 12.5% ± 6.3. Fish fed the base plus 80 mg $CuSO_4$ kg^{-1} for 4 weeks had a mean survival of 40.5% ± 8.1. There was a significant effect after 4 weeks with fish fed the base plus 80 mg $CuSO_4$ kg^{-1} mg not with 40 mg kg^{-1}.

Keywords: columnaris disease, copper supplementation, channel catfish, survival

Introduction

Copper is an essential mineral for both plant and animal metabolism. In animals, copper is vital for haemoglobin production, bone formation and is crucial for the activity of a suite of enzymes including various metalloenzymes, cytochrome oxidase, superoxide dismutase, lysyl oxidase and tyrosinase (Murai, Andrew & Smith 1981). Several studies have characterized the effects of elevated dietary copper supplementation in animal diets including beef and dairy cows, poultry and fish (Murai *et al.* 1981; Gatlin & Wilson 1986; Chirase, Hutcheson & Thompson 1991; Xin, Waterman, Hemken & Harmon 1991; Scaletti, Trammel, Smith & Harmon 2003; Samanta, Biswas & Ghosh 2011; Scaletti & Harmon 2012).

Prior research has investigated a wide range of possible influences of copper supplementation on growth, enzyme activity, lipid profiles, resistance to disease and immune function. Supplementation with copper has been shown to enhance neutrophil function or improve resistance to a disease challenge in dairy and beef cattle (Chirase *et al.* 1991; Xin *et al.* 1991; Scaletti *et al.* 2003). Chelated copper supplements are thought to enhance the immune response of early lactation in dairy cows, including an increased antibody titre response to vaccination (Nemec *et al.* 2012). Copper supplementation in the diet was found to be commercially beneficial to increasing production beyond the cost of the supplementation for poultry at 150 mg Cu kg^{-1} (Samanta *et al.* 2011). Supplemental copper improved feed efficiency in cattle consuming diets containing 60% distiller's dried grains with solubles; however, effects of copper on rumen sulphur metabolism were minimal, even when supplemented at twice the maximum tolerable limit for beef cattle (NRC, 2001).

There are established differences in copper sensitivity across a range of terrestrial and aquatic animals that has been linked to metallothionein induction and tissue copper concentrations (De Boeck, Huong Ngo, Campenhout & Blust 2003). The dietary requirement of channel catfish (*Ictalurus punctatus*) fingerlings has been estimated at 5 mg Cu kg^{-1}, and is typically added to the diet as copper sulphate ($CuSO_4$; Gatlin & Wilson 1986; Tucker & Hargraves 2009). Dietary supplementation of up to 600 mg Cu kg^{-1} did not adversely affect rainbow trout (*Oncorhynchus mykiss*) (Lanno, Slinger & Hilton 1985). Differences in copper distribution at the subcellular levels of gills and liver in some fish species strongly reflect their capacity to handle excess copper and are one of the factors in their varying sensitivity to copper (Eyckmans, Celis, Horemans, Blust & De Boeck 2011). Acute dietary toxicity from copper leads to lesions in the foregut and gastrointestinal haemorrhage in trout (Handy 2003). The chronic lethal dose for rainbow trout – one of the more sensitive species tested is higher than 10 g Cu kg^{-1} body weight with sublethal effects occurring between 500 and 1000 mg Cu kg^{-1} body weight (Lanno *et al.* 1985; Handy, Sims, Giles, Campbell & Musonda 1999; Handy 2003).

Columnaris disease, caused by *Flavobacterium columnare*, continues to be one of the most detrimental bacterial diseases of channel catfish commercially grown in the United States (USDA, 2010). Bacterial challenges with *F. columnare*, as well as *Edwardsiella ictaluri*, following exposure to a prophylactic dose of waterborne $CuSO_4$ resulted in copper-exposed fish to be more resistant to these diseases than unexposed fish (Griffin & Mitchell 2007; Farmer, Beck, Mitchell & Straus 2013). Building upon these previous reports, in this study we investigated the effects of copper-supplemented diets fed to channel catfish on susceptibility to columnaris disease.

Methods

Fish husbandry

Channel catfish fingerlings were maintained indoors at the USDA/ARS – Harry K. Dupree Stuttgart National Aquaculture Research Center (SNARC; Stuttgart, Arkansas) in 250-L fibreglass stock tanks. Fish were fed and monitored daily during the acclimation period and feeding trial. The turnover rate of the flow-through well water (24°C ± 2) was once every 2 h. Average weight of fingerlings was 6.7 ± 1.1 g (mean ± SD) at the beginning of the feeding study. Animal care and experimental protocols were approved by the SNARC Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

Copper sulphate diet supplementation

A commercial catfish fry/fingerling crumbled diet was obtained from FishBelt Feed (Moorhead, MS, USA) containing a minimum 35% protein, 2.5% fat and 0.7% phosphorus and a maximum of 7% fibre. The crumbles were passed once through a hammer mill yielding a fine powder. Ground diet was then rehydrated in a Hobart mixer (Model A-200; Hobart, Troy, OH, USA) using deionized water containing the desired $CuSO_4$ concentration. Once the diet mash had reached approximately 30–40% moisture and uniform consistency, the diets were cold-pressure pelleted in a commercial pasta extruder (Model LAB2V41, Ambrette Machinery Corp., Brooklyn, NY, USA). The diets were then oven dried to 90% dry matter and stored at −4°C until fed. Samples of the finished diets were analyzed for copper concentrations by standard methods (APHA 2005) using inductively coupled plasma (ICP) spectroscopy (Arkansas Analytical, Inc., Little Rock, AR, USA). All channel catfish fingerlings were fed the basal diet during the 7 day acclimation period prior to initiating the feeding trial. After acclimation, each of nine tanks of fish were randomly assigned and fed one of the following test diets: the basal diet (unsupplemented), basal + 40 mg $CuSO_4$ kg^{-1} diet and basal + 80 mg $CuSO_4$ kg^{-1} diet. Each tank contained 800 fish and fish were offered feed at 3% body weight daily (160.8 g feed per tank). Feed weight was adjusted for dry matter per individual diet, and feeding behaviour was observed and recorded. A subsample of 20 fish was weighed and measured individually from each tank after two and 4 weeks of feeding. Feeding rates were adjusted at 2 weeks for growth and the removal of the subsampled fish. At 2 weeks and at 4 weeks, 150 fish and 90 fish, respectively, from each diet were removed and challenged with *F. columnare*, as described below, to assess the effect of diet copper supplementation on disease survival.

Challenge method

Fish were reallocated to the challenge aquaria at a rate of 50 fish per 18 L tank holding 10 L water in experiment 1 (2 week challenge) and 30 fish per tank in experiment 2 (4 week challenge). There were three replicates per treatment and one unchallenged control from each diet for a total of 12 aquaria. Twenty-five fish were weighed and measured prior to stocking tanks receiving the bacterial challenge. Fish were then exposed to *F. columnare* isolate LV-359-01, an isolate which was previously shown to produce consistent mortality in the low-flow system (Mitchell & Farmer 2010; Farmer, Mitchell & Straus 2011; Farmer *et al.* 2013). The isolate was retrieved from the −80°C freezer and streaked on Ordal's medium (Anacker & Ordal 1955). After 48 h, the isolate was dislodged from the agar using a sterile cotton swab and inoculated into 5 mL of *F. columnare* Growth Medium (FCGM; Farmer 2004). This suspension was incubated at 28°C for 24 h, and the 5 mL starter culture was used to inoculate 1 L of FCGM. The inoculated broth was incubated for 24 h at 28°C in an orbital shaker incubator at 200 rpm. When the bacterial growth reached an absorbance of 0.68 at 550 nm (approximately 4.0×10^{10} colony forming units [CFU] mL^{-1}), the flask was removed and placed on a stir plate at room temperature. A 10 mL aliquot was removed for serial dilution and CFU enumeration. In all challenges, 125 mL of bacterial suspension was added to tanks receiving the challenge dose (5.0×10^8 CFU mL^{-1} per tanks), and sterile broth was added to the control tanks.

Temperature and dissolved oxygen were measured daily with an YSI Pro20 (YSI, Inc., Yellow Springs, Ohio, USA). The mean dissolved oxygen concentration was 5.6 ± 1.2 mg L^{-1} and water temperatures ranged from 26.2 to 27.5°C. Total ammonia nitrogen (TAN) concentrations were determined in each tank with a Hach DR/4000V spectrophotometer using the Nessler Method 8038 (Hach Company, Loveland, CO, USA). An Orion 720A pH meter (Fisher Scientific, Waltham, MA, USA) was used to measure pH (7.5–8.2) at the beginning of the study. Standard titration methods (APHA 2005) were used to measure total alkalinity (213 mg L^{-1}) and total hardness (112 mg L^{-1}). Fish were observed daily for clinical signs associated with columnaris disease. Fish unable to maintain neutral buoyancy were considered moribund and removed for dissection and bacteriological sampling. Fish were not fed during the challenge experiment.

Clinical observation and histology

Fish were observed twice daily for signs of disease and observations were recorded. Mortalities were recorded twice daily and analyzed as a function of time in half day intervals. Intestines were sampled from five fish from each treatment group after 2 weeks and 4 weeks on the test diets. Approximately, 1 cm of the anterior intestine was removed and immediately fixed in 10% neutral buffered formalin. After 48 h in the fixative, the sampled tissues were transferred to 70% isopropanol and stored until routine paraffin embedding with a Leica TP1020 tissue processor (Buffalo Grove, IL, USA). Embedded tissues were then sectioned (5-6 μm) with a Leica RM2135 microtome, mounted on glass slides and stained with haematoxylin and eosin for pathological evaluation.

Copper tissue analysis

Samples were collected after 4 weeks of feeding from fish fed the basal diet and the diet supplemented with 80 mg $CuSO_4$ kg^{-1} diet to investigate whether bioaccumulation may have occurred. Samples included the right second gill arch, anterior intestine, liver and (≈1 cm^2) of muscle and skin to be analyzed for copper concentration. Samples were placed in −80°C freezer until analyzed. Copper concentrations were measured by standard methods (APHA 2005) using inductively coupled plasma (ICP) spectroscopy.

Statistical analysis

Survival data were analyzed using SigmaPlot 11 (San Jose, CA, USA) by Kaplan–Meier Log Rank Survival Analysis, and all pair-wise multiple comparisons used the Holm–Sidak method with type three adjusted *P*-values. Each experiment was analyzed separately using 'treatment' as the fixed effect and 'replicates' as the random effect. One way ANOVA was used to compare copper concentrations measured in tissue samples by diet. Treatment effects were considered significant at $P \leq 0.05$.

Results

Copper diet and tissue analysis

The base concentration of copper in the unsupplemented (basal) diet was 18.4 mg Cu kg^{-1} diet. The 40 and 80 mg CuSO4 kg^{-1} diets supplemented diets contained 28.6 mg Cu kg^{-1} diet and 37.7 mg Cu kg^{-1} respectively; the calculated copper sulphate and final supplementation levels are included in Table 1. The levels of copper in the tissues sampled are shown in Table 2; no statistical difference was found in tissue copper concentrations between fish on any diet.

Table 2 Mean copper concentrations in $\mu g\ g^{-1} \pm$ SEM in tissues sampled from channel catfish fed basal diet or a diet supplemented with 80 mg $CuSO_4$ kg^{-1} diet as determined by ICP analysis

	Diet	
Tissue	Basal	+80 mg $CuSO_4$ kg^{-1}
Gill	1.79 ± 0.83	1.43 ± 0.66
Intestine	4.08 ± 0.67	6.02 ± 2.65
Liver	17.92 ± 10.12	17.9 ± 8.63
Muscle	3.34 ± 1.33	2.23 ± 0.50

ICP, inductively coupled plasma

Clinical signs and histology

Moribund fish in challenge tanks displayed signs consistent with an *F. columnare* infection. Gross pathologies were also typical; the skin of moribund fish initially had discrete depigmented areas that became multifocal to diffuse as the infection progressed. Dermal ulceration and cutaneous sloughing exposed the underlying muscles, and severely frayed fins were observed. Several gill samples were observed to have focal to multifocal branchial necrosis with yellowish pigment. No internal gross changes or lesions were observed. If present, a maximum of three moribund or dead fish were sampled from each tank per day. At the conclusion of experiment 1 and 2, 10 putative positive isolates were confirmed using species specific PCR primers (Panangala, Shoemaker & Klesius 2007). Histological evaluation of intestines revealed that no microscopic lesions were associated with elevated levels of dietary copper (data not shown).

Survival of fish challenged after 2 weeks on supplemented diets

Survival after challenge was significantly higher in channel catfish fed diets with $CuSO_4$ supplements for 2 weeks (Fig. 1). This increase in survival appears to be dose-dependent, meaning fish receiving the diet with more copper had significantly higher survival. The mean per cent survival (±SEM) for fish fed the basal diet for 2 weeks and then challenged was 2.0 ± 1.1%. Fish fed the basal + 40 mg $CuSO_4$ kg^{-1} diet prior to challenge had a mean survival of 22.0 ± 11.0%. Fish fed the basal + 80 mg $CuSO_4$ kg^{-1} diet prior to challenge had a mean survival of 29.3 ± 13.4%. Fish that had been fed copper-supplemented diets had significantly higher rates of survival than fish fed the basal diet ($P \leq 0.05$). A significant difference was also found between the + 40 mg $CuSO_4$ kg^{-1} and the + 80 mg $CuSO_4$ kg^{-1} supplemented groups. No fish died in the three non-challenged tanks consisting of one tank from each of the three test diets. Mortality persisted for 5 days, but the majority of fish deaths occurred by day 3. The study was concluded on day 7, after 2 days of no mortalities.

Survival of fish challenged after 4 weeks on supplemented diets

The mean per cent survival for fish fed the basal diet for 4 weeks and then challenged was 28.3 ± 9.0%. Fish fed the basal + 40 mg $CuSO_4$ kg^{-1} diet prior to

Table 1 Mean concentrations (mg kg^{-1} diet ± SEM) of measured copper, calculated copper sulphate and supplementation level; cu concentration measured in the test diets by ICP analysis

Diet	Measured Cu	$CuSO_4$	*Supplemental $CuSO_4$
Basal	18.35 ± 0.92	72.1	0
+40 mg $CuSO_4$ kg^{-1}	28.6 ± 0.28	112.4	40.3
+80 mg $CuSO_4$ kg^{-1}	37.7 ± 0.57	148.1	76.0

*Measured Cu × 3.93 = $CuSO_4$ – Basal $CuSO_4$ = Supplemental $CuSO_4$.
ICP, inductively coupled plasma

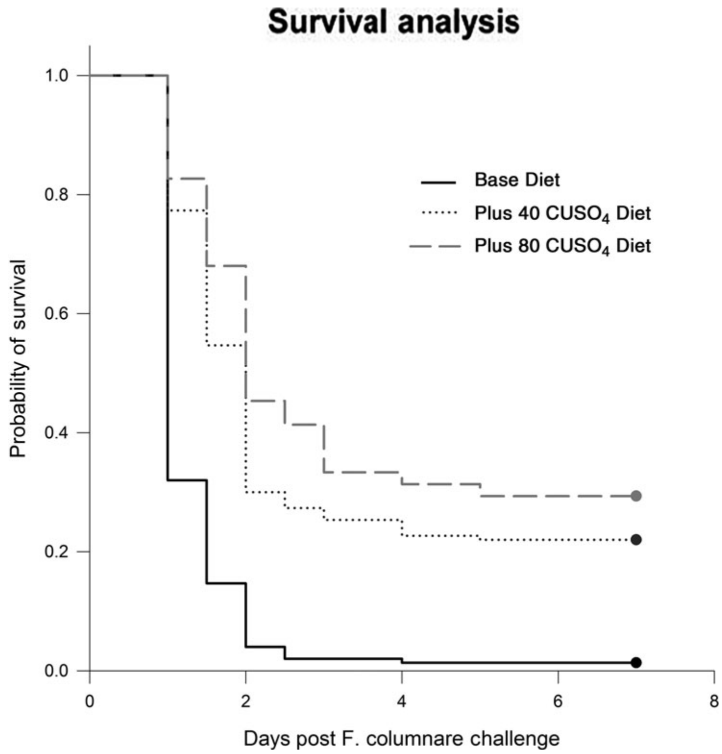

Figure 1 Kaplan–Meier survival curve of channel catfish fed one of the three levels of copper-supplemented diets for 2 weeks than challenged with *F. columnare*. Dietary treatments included: (1) basal (unsupplemented) diet, (2) basal diet supplemented with 40 mg $CuSO_4$ kg^{-1} and (3) basal diet supplemented with 80 mg $CuSO_4$ kg^{-1}.

challenge had a mean survival of 12.5 ± 6.3%. Fish fed the basal + 80 mg $CuSO_4$ kg^{-1} diet prior to challenge had a mean survival of 40.5 ± 8.1%. There was no significant difference in survival between fish fed the + 40 mg $CuSO_4$ kg^{-1} diet and those fed the base diet. There were, however, significant differences ($P \leq 0.05$) between the two copper-supplemented groups as well as a significant difference between the + 80 mg $CuSO_4$ kg^{-1} diet and the basal diet group. No fish died in the three non-challenged tanks consisting of one tank from each of the three test diets. Mortality persisted for 5 days, but the majority of fish deaths occurred by day 3. The study was concluded on day 7, after 2 days with no mortalities. Survival curves are depicted in Fig. 2.

Growth

All three diets were consumed entirely at each feeding. A single mortality was observed during the 4-week feeding period that was not associated with the challenge. The average fish weights at the start of the study were 6.7 g. Average fish weights on base diets at 2 and 4 weeks were 9.0 and 12.6 g respectively. Average fish weights on basal + 40 mg CuSO4 kg^{-1} for 2 and 4 weeks were 9.5 and 12.9 g respectively. Average fish weights for basal + 80 mg CuSO4 kg^{-1} for 2 and 4 weeks were 10.7 and 12.5 g respectively. There was no difference in growth over the course of the study.

Discussion

The dietary requirement of copper for channel catfish fingerlings has been previously estimated at 5 mg Cu kg^{-1} diet (Gatlin & Wilson 1986). There are no documented cases of copper deficiency in commercially raised channel catfish, suggesting that current requirement estimate supports the physiological and nutritional needs of the fish. The results of this study corroborate the current requirement estimate since no differences in growth or feed conversion were observed among our dietary treatments. These results do suggest, however, that elevating dietary copper levels may influence disease-related survival, at least temporarily.

The survival rate of fish challenged after being fed the diet supplemented with 80 mg $CuSO_4$ kg^{-1} diet compared to the control (basal) diet suggests

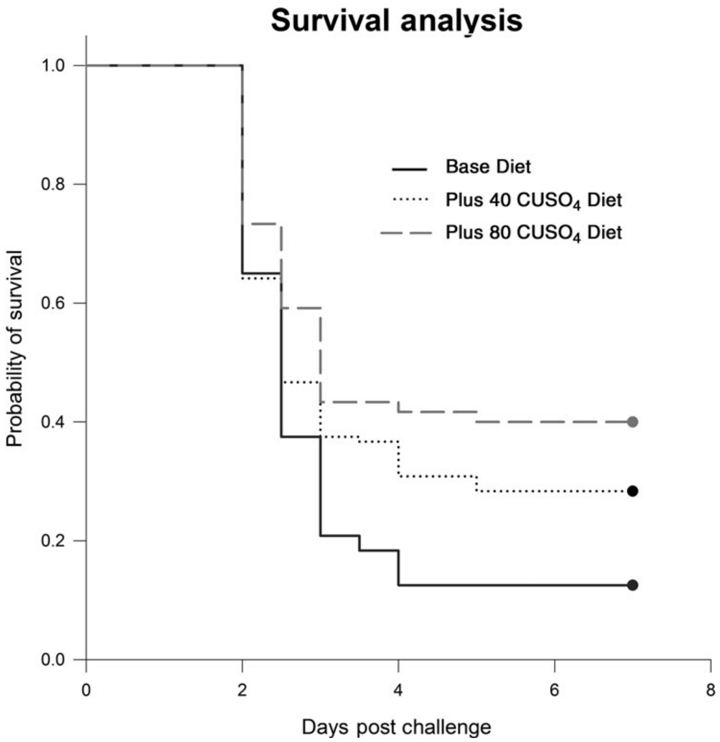

Figure 2 Kaplan–Meier survival curve of channel catfish fed one of the three levels of copper-supplemented diets for 4 weeks than challenged with *F. columnare*. Dietary treatments included: (1) basal (unsupplemented) diet, (2) basal diet supplemented with 40 mg $CuSO_4$ kg^{-1}, and (3) basal diet supplemented with 80 mg $CuSO_4$ kg^{-1}.

that some level of protection to columnaris disease was afforded. In both experiments, fish fed the + 80 mg $CuSO_4$ kg^{-1} diet performed better than those in the control or those fed the + 40 mg $CuSO_4$ kg^{-1} diets. It appears that the marginal protection acquired with the +40 mg $CuSO_4$ kg^{-1} diet was transient and not apparent at week four. The mechanism of diet-related copper protection has yet to be defined; however, protection was also apparent when fish were exposed to waterborne $CuSO_4$ and subsequently challenged with *F. columnare* (Farmer *et al.* 2013).

The mechanism of copper-induced disease resistance is not well characterized, particularly in fish. Previously, it was determined that a concentration of 4.2 mg L^{-1} inhibit the growth of *F. columnare in vitro* (Darwish, Mitchell & Straus 2012). It is possible that an increased dietary copper inhibited the ability of the bacterium to go systemic and as a result improve survival. However, based on the mean copper concentrations from basal diet compared to supplemented diet with 80 mg $CuSO_4$ kg^{-1}, no significant difference was apparent from the tissues sampled Table 2. However, copper driven inhibition of the bacterium cannot be ruled out. A temporary spike in copper concentration post feeding could occur or concentrations could be elevated in other tissues possibly by blood or mucous.

It is plausible that the afforded protection could be related to changes in the fish microbiota, particularly on the skin and gills since they are the primary source of infection. There is a varied tolerance to copper among different bacterial species generally attributed to the copper-exporting ATPase-type pump (Hodgkinson & Petris 2012). Therefore, one hypothesis is that elevated copper will increase the number of copper-tolerant species in the microbiota and induce a response that improves disease resistance. More likely, the mechanism is multifaceted, particularly when copper is in contact with gut mucosal surfaces since the gut is not a primary site of columnaris infection and instead another component of the fish's immune system (Beck & Peatman 2015). Copper has been shown to have immunoregulatory properties in some species (Chirase *et al.* 1991; Xin *et al.* 1991; Scaletti *et al.* 2003). Supplementation with copper or zinc enhances neutrophil function or improves response to a disease challenge in dairy and beef cattle (Chirase *et al.* 1991; Xin *et al.* 1991; Scaletti *et al.* 2003).

The immune system has a copper-mediated bactericide in activated macrophages where copper is used to generate superoxides that kill the bacteria once it has been engulfed by the macrophage (Hodgkinson & Petris 2012). In fact, a mutation in the copper export system in *Mycobacterium tuberculosis* attenuated the isolate in guniea pigs (Ward, Abomoelak, Hoye, Steinberg & Talaat 2010). Further evidence is provided by studies on mice infected with *M. tuberculosis* deficient in the membrane channel protein where the infection rate was reduced 100 folds when the $CuSO_4$ was added to their drinking water. Because the reduced infection was based on the ability of the bacteria to export copper, it is subject to the availability of excess copper in the host (Wolschendorf, Ackart, Shrestha, Hascall-Dove, Nolan, Lamichhane, Wang, Bossmann, Basaraba & Niederweis 2011). It is also well documented that copper levels in serum were significantly elevated in response to inflammation (Conforti, Franco, Milanino, Totorizzo & Velo 1983; Akçil, Yavuz & Koçak 2003), so it stands to reason that if a host animal had access to and could tolerate excess copper, it could mount a better inflammatory response.

The current supplementation rate was based on the 20 mg Cu kg^{-1} diet measured in the basal feed, which is four times the estimated nutritional requirement (Gatlin & Wilson 1986). The supplemented feed was 1.5× and 2× the basal level, so there could be some optimization of the supplementation level and feeding regimen needed to illicit the maximum response. Since the survival rate in fish fed the 80 mg $CuSO_4$ kg^{-1} diet was significantly higher than those fed the 40 mg $CuSO_4$ kg^{-1} diet, higher supplementation rates should be investigated to determine maximum efficacy. However, caution must be advised until any long-term negative impacts have been ruled out. Copper can be toxic due to its ability to generate reactive oxygen species (Hodgkinson & Petris 2012). These negative impacts and any possible beneficial impacts would need to be validated in a commercial setting prior to potential adoption by the industry. At the times sampled (weeks 2 and 4), no elevated levels of copper were found in selected tissues, which should reduce concerns about food safety but should be further confirmed.

The results of this study suggest that the copper-mediated increase in survival may only be temporary. Considering these limitations, a diet containing elevated levels of copper may not be feasible as a stand-alone ration. Instead, such a diet could be better utilized as a management practice for producers; for instance, feeding an elevated rate of copper when there is an increased risk of an outbreak such as prior to seining, grading or seasonal temperature changes. One obvious advantage to using copper in the diet would be the negligible amount of copper being released into the environment. With the rates of inclusion in this study, the amount used in a ton of feed is marginal compared to what is typically used in a water application to a commercial pond. Further, in contrast to a pond treatment, the addition of $CuSO_4$to the diet is not cost prohibitive, as 2 lbs of $CuSO_4$ would only cost an extra $4 ton^{-1} at current prices.

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